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## TRANSLATION CERTIFICATE

OFFICIAL

I, Takeo Ohashi, President, Ohashi High Technology Corporation, the Empire State Building, 350 Fifth Avenue, Suite 607, New York, NY 10118-6399, hereby declare that I have competent knowledge of the Japanese and English languages, and that I have reviewed the accompanying English translation of Japanese patent application publication No. H1-172332. I certify that the translation is an accurate representation of the Japanese patent application publication No. H1-172332.

I declare under penalty of perjury that the foregoing is true and correct.  
Executed on September 10, 2003.

CERTIFIED by the said Takeo Ohashi.

at 350 Fifth Avenue, Suite 607, New York, NY 10118  
this 10th day of September, 2003.

A handwritten signature in dark ink, appearing to read "Takeo Ohashi", is written over a horizontal line.

Takeo Ohashi

President

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54)	Title of Invention:	Agent to Prevent and Treat Bovine Mastitis	
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### Specification

1. Title of Invention:

An Agent to Prevent and Treat Bovine Mastitis

2. What is Claimed is:

Claim 1) An agent to prevent and treat bovine mastitis caused by staphylococci which contains one or more galenicals which are selected from a group comprising of kanzo, oren, ogon, kohboku, tanjin, chimo, chouji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, bogaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou.

Claim 2) The agent to prevent and treat bovine mastitis according to Claim 1, wherein the galenicals are in their original powder form.

Claim 3) The agent to prevent and treat bovine mastitis according to Claim 1, wherein the galenicals are employed in a form of a galenical extract solution which is obtained through extracting the galenicals with an organic solvent, a mixture of water and organic solvents or water, and/or in a form of effective galenical constituents obtained from such a galenical extract solution.

Claim 4) The agent to prevent and treat bovine mastitis according to Claim 1, wherein the galenicals are mixed in feed.

Claim 5) A method to prevent and treat bovine mastitis caused by staphylococci which comprises administering an effective dosage of one or more galenicals which are selected from a group comprising of kanzo, oren, ogon, kohboku, tanjin, chimo, chouji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, bogaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou.

Claim 6) The method to prevent and treat bovine mastitis according to Claim 5, wherein the galenicals are in their original powder form.

Claim 7) The method to prevent and treat bovine mastitis according to Claim 5, wherein the galenicals are employed in a form of a galenical extract solution which is obtained through extracting the galenicals with an organic solvent, a mixture of water and organic solvents or water, and/or in a form of effective galenical constituents obtained from such a galenical extract solution.

Claim 8) The method to prevent and treat bovine mastitis according to Claim 5, wherein the galenicals are mixed in feed and then administered.

3. Detailed Explanation of the Invention

[Field of Industrial Application]

The present invention pertains to an agent to prevent and treat bovine mastitis, and a method to prevent and treat bovine mastitis.

In more detail, it pertains to an agent containing galenicals to prevent and

treat bovine mastitis caused by staphylococci and a method to prevent and treat the disease by administering galenicals.

[Prior Art Technologies]

Bovine mastitis with staphylococci is caused by staphylococci infection, especially staphylococcus aureus infection.

This disease causes reduction in milk production, degradation in milk quality, shorter milk production lifetime, infection of other cows and so forth, which result in large losses to the dairy industry. Furthermore, contamination of milk by the bacteria causes adverse impacts to public health.

Despite the fact that bovine mastitis is such a serious infectious disease, we have very few effective measures against it. Some countermeasures are taken such as proper management for raising cows. However, in many cases, the disease is not preventable.

Further, at present, methods to prevent and treat this disease, such as administering antibiotics and other antibacterial agents mixed with feed are implemented. However, especially in recent years, problems have aroused such as the appearance of drug-resistant bacteria and antibiotic residues and the like in animals. Thus, public opinion against the administration of antibiotics and the like to farm animals is gaining momentum. Therefore, it is desired to develop a preventive and treatment method and a preventive and treatment agent for this disease, without employing antibiotics and the like.

[Means to Resolve the Issues]

The inventors conducted research to solve the aforementioned issues. As a result, the inventors found that some kinds of galenicals have efficacy to suppress this disease, thus achieving this invention.

In other words, the purpose of the present invention is to provide a prevention and treatment agent of bovine mastitis caused by staphylococci, and a prevention and treatment method of the same disease.

The present invention provides an agent to prevent and treat bovine mastitis caused by staphylococci which contains one or more galenicals which are selected from a group comprising of kanzo, oren, ogon, kohboku, tanjin, chimo, chouji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, bogaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou.

The present invention also provides an agent to prevent and treat bovine mastitis caused by staphylococci, wherein the galenicals are in their original powder form, or wherein the galenicals are employed in a form of a galenical extract solution which is obtained through extracting the galenicals with an organic solvent, a mixture of water and organic solvents or water, and/or in a form of effective galenical constituents.

The present invention also provides an agent to prevent and treat bovine mastitis caused by staphylococci, wherein the galenicals are mixed in feed.

Furthermore, the present invention provides a method to prevent and treat bovine mastitis caused by staphylococci which comprises administering an effective dosage of one or more galenicals which are selected from a group comprising of kanzo, oren, ogon, kohboku, tanjin, chimo, chouji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, bogaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou.

The present invention also provides a method to prevent and treat bovine mastitis caused by staphylococci, wherein the galenicals are in their original powder form, or wherein the galenicals are employed in a form of a galenical extract solution which is obtained through extracting the galenicals with an organic solvent, a mixture of water and organic solvents or water, and/or in a form of effective galenical constituents.

The present invention also provides a method to prevent and treat bovine mastitis caused by staphylococci, wherein the galenicals are mixed in feed and then administered.

As for kanzo, roots or stems of *Glycyrrhiza glabra* Linad var. *gladulifera* Regel et Herder are employed in its natural form, or after the cork cambium is removed. Kanzo contains glycyrrhizin, rikirrhizin, glucose and asparagines and so forth as its constituents and is used by humans to help with relaxation and used as an antispasmodic agent and a phlegm removal agent.

As for oren, roots and stems of *Coptis japonica* Makino are employed after fine roots are burned and removed from its roots. Oren contains veruberin, balmacine and so forth as its constituents and is used by humans as a bitter tonic for peptic health and an agent for intestinal disorders.

As for ogon, the roots of *koganebana* (*Scutellaria baicalensis* Georgi) are employed after cork cambium is removed. Ogon contains ogonin and baicalein as its constituents and is used by humans as an anti-inflammation agent and an agent for fever.

As for koboku, the bark of the tree trunk and branches of honoki (*Magnolia obovata* Thunberg) is employed. Koboku contains magnocararin and a camellia oil as its constituents and the camellia oil contains matirole and magnol as its major constituents. Humans use it as an astringent diuretic to treat chest and abdominal fullness as a phlegm removal agent, stomachaches and phlegm and coughing.

As for tanjin, the roots of *Salvia sillilorrhiza* Bunge are employed. Tanjin contains tangenon I and II, and cryptotangenon as its constituents. Tanjin is used by humans to enhance vigor, and treat irregular menstruation as a menstruation regulating agent and feminine medication, uterus bleeding, stomachaches and various injuries.

As for chimo, the roots and stems of hanasuge (*Asomanhena asphodeoides* Bunge) are employed. It contains saponin, steroid saponin, tannin and so forth as its constituents. It is used by humans to treat inflammation, fever and vomiting, to promote urination and to relieve pain.

As for choji, the buds of *Bugenia caryophyllata* Thuab are employed just before blooming. Choji contains oigenol and acetyl oigenol and so forth as its constituents. It is used by humans to improve digestive functions and to rid parasites. It is also used as a spice and used in cosmetics.

As for kujin, the roots of kurara (*Sophora Augustifolia* Siebold et Zuccarini) are employed in its natural form, after removing most of its cork cambium and cutting it longitudinally or transversely. Kujin contains matrine as its constituent, and is used by humans to improve stomach health, to promote urination, to reduce fevers and pain, and torid parasites.

As for keihi, keiju (*Ciana sosus* Cassia Bluso) is employed after its dry bark on the trunk and branches and some of the cork cambium are removed. It contains cinnamaldehyde and cinnamyl acetate as its constituents and is used by humans to induce perspiration, to treat fever and pain, to improve stomach functions, to treat intestinal disorder and to rid parasites.



As for biwayo, leaves of biwa (*griboirya japonica* Lindley) are employed after as many fine hairs as possible are removed. Biwayo contains glucose, sucrose, fructose, maltose and so forth as its constituents. It is used by humans to treat and prevent the suffering from heat as a refreshment and used as stomach tonic.

As for hop, ripe ears of hops (*Humulus Lupulus* Linne) are employed. Hop contains tannin, humulone, lupulone and so forth, and is a necessary ingredient for brewing beer to render a bitter taste and aroma.

As for yobaihi, the bark of yamamomo (*Myrica rubra* Siebold et Zuccarini) is employed. Yobaihi contains tannin, myricetin, myricitrin and so forth, and is used by humans to treat diarrhea as an astringent agent. It is also effective for treating contusions.

As for enmeiso, the stems and leaves of hikiokoshi (*Isodon japonica* Hara) or kurobana-hikiokoshi (*Isodon trichocarpus* Kudo) are employed. Enmeiso contains prectolentin and enmein as its constituents. It is used by humans to treat indigestion, lack of appetite, stomachaches and so forth and as a bitter tonic for better stomach health.

As for gobaishi, is a gall which appears mainly on nurude (*Rhus javanica* Linne) caused by scars from bites by nurudeno-mimifushi-aburamushi (*Melaphis chinensis* J. Bell). Gobaishi contains tannin, gallic acid, fat, resins and so forth as its constituents. It is used as a raw material to produce Gobaishi tincture, tannic acid, gallic acid and pyrogallic acid.

As for sanshu, the seeds extracted from ripe sanshu (*Cornus officinalis* Sichoid et Zuccarini) are employed. Sanshu contains crystalline organic acids, gallic acid, mallic acid, tartaric acid and so forth as its constituents. It is used by humans as a nourishing and health tonic and as an astringent agent.

As for shakuyaku, the roots of shakuyaku (*Paeonia albiflora* Pallas var. *trichocarpa* Bunge) are employed. Shakuyaku contains benzoic acid, resins and so forth as its constituents. It is used by humans as an astringent agent, a relaxation agent, and a pain relieving agent, and as an antispasmodic agent.

As for kinnoshi, the accessory fruits of naniwaibara (*Rosa laovigara* Michaux) are employed. Kinnoshi contains citric acid, mallic acid, tannin, saponin, vitamin C and so forth as its constituents. It is used by humans to stop diarrhea and polyuria.

As for chiyu, the dried roots of waremoko (*Sanguisorba officinalis* L.) are employed. Chiyu contains tannin (17%) and saponin as its constituents. It is used by humans to treat hematemesis, hemoptysis and excess menstruation as a hemostatic astringent agent.

As for maou, the aboveground stems of maou (*Ephedra sinica* Stapf) are employed. Maou contains l-ephedrin, d-pseudo-ephedrin and so forth as its constituents. It is used by humans to treat coughing and as an agent to induce perspiration and urination.

As described above, each of the galenicals which are used in the present invention; i.e. kanzo, oren, ogon, koboku, tanjin, chimo, choji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, bobaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou, have been used singly or in combination to prevent and treat human diseases in prior arts. However, no effort has been made to use them for the purpose of preventing and treating diseases of cows, especially for the purpose of preventing and treating infectious diseases of cows.

The present inventors took notice of the effects of the aforementioned galenicals, and as a result of various research, have discovered that administering one of the aforementioned galenicals or a combination of two or more thereof to cows is extremely effective for prevention and treatment of bovine mastitis which is inflicted by staphylococci, especially staphylococcus aureus. Thus, the present inventors completed the present invention.

This is to say, as described below, it became clear that the galenicals specified in the above and their extracts have bacteriostatic and biocidal effects which are specific to the bacterium, staphylococcus aureus, *in vitro* and *in vivo*. As a result of this suppressive property against the bacterium, staphylococcus aureus, administering these galenicals enabled the present inventors to prevent and treat the cows' disease, bovine mastitis, which is caused by staphylococcus aureus.

It had not been anticipated that these galenicals and their extracts are effective in treating the disease which is unique to cows. The present inventors made this clear for the first time.

These galenicals are used by administering them in an original powder form, in an extract solution form, or as an effective galenical constituent, or by adding these to animal feed, or by processing them into an agent form such as a liquid agent form, a dispersed agent form, a semi-solid agent form, a powder agent form, a solid form agent form, a leachate agent form, an injection agent form and so forth.

An extract solution is extracted by means of solvent extraction using, for example, water, an organic solvent or a mixture of water and an organic solvent. It is employed as is, after being condensed or diluted, or after its solvent is removed.

As examples of an organic solvent, methanol, ethanol, n-propanol, n-butanol, acetone, ethyl acetate, ether, methylene chloride, chloroform, benzene, carbon tetrachloride, petroleum ether and so forth can be employed. Methanol and ethanol are especially preferred. One of these organic solvents can be employed or two or more can be combined and employed as a mixture.

Galenicals may be immersed in a cold or warm solvent in order to perform solvent extraction. A temperature range could be between 15~25 centigrade to conduct a cold immersion and 35~45 centigrade to conduct a warm immersion. The extraction time period varies depending on the extraction temperature, but in general it takes about 5 days or until soluble constituents are sufficiently dissolved.

Effective constituents of galenicals, for example, can be mixed and extracted further by applying various solvents to the aforementioned extract solution. Fractions in which the constituents are dissolved in the solvents are separated. The fractions are left for the solvents to leave. Thus obtained materials are employed as effective fractions, in other words as effective constituents.

Examples of solvents employed for such instances include non-polar solvents such as lower aliphatic ethers (diethyl ether, ethylmethyl ether and so forth); lower halogen alkanes (chloroform, dichloro ethane, dibromoethane and so

forth); benzenes (benzene, toluene, xylene and so forth); and other petroleum solvents (petroleum ether, petroleum benzene, ligroin and so forth).

In addition, an aqueous solution which remains after removing the effective fractions is utilized by using water and a certain organic solvent which is saturated with water for mixing and extracting. After washing with water, fractions which transfer to the remaining organic solvent layers are left for the

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solvents to leave. The residues are employed as effective fractions, in other words effective constituents. These effective fractions are different from the effective fractions which are previously described in physical and chemical properties and the constituents which can be detected are also different.

The amount to be administered varies in a range from 0.001 mg/body weight kg/day ~ 100 g/body weight kg/day in the original weight of galenicals. An amount of an agent for prevention is different from that of the agent for treatment, and generally the amount to be administered is larger for the latter.

The method of giving it is either oral or non-oral administration. As non-oral administration, intramuscular, intra stomach, transdermal, nasal and vein administration can be used.

#### [Advantages of the Invention]

As stated above in detail, the present invention provides effective agents and methods to prevent and treat bovine mastitis caused by staphylococci.

The prevention and treatment agents according to the present invention employ galenicals or extracts derived therefrom as the major agent constituents. Thus, there is no problem at all, such as appearance of resistant bacteria and side effects, which are issues with antibiotics. The agents can be administered without concerns.

Moreover, there is no need to be concerned over the adverse effects of residual agents on the human body, and therefore it is appropriate to administer the agents to cows for meat consumption to prevent and treat bovine mastitis caused by staphylococci.

Furthermore, the same disease can be prevented and treated at much cheaper costs than those when antibiotics and so forth are employed.

Further explanation of the present invention is offered below using Test Examples and Examples.

## Test Example 1.

Susceptibility of staphylococcus aureus to each of the galenicals was tested.

Each galenical was added to 50% aqueous ethanol having a volume 7 times that of the galenical and extracted for 16 hours to give an extract in alcohol.

Disks with a diameter of 8mm were each soaked with 25 $\mu$ l of the respective extract and dried. Each disk was placed on a heart infusion agar to which staphylococcus aureus was applied. After incubating for 18 hours at 37 centigrade, the diameter of each blocking circle was measured.

Next, Table 1 below shows the test results.

Table 1

<u>Names of galenicals</u>	<u>Blocking disk (mm) by extract</u> <u>Using alcohol</u>
Kanzo	12
Oren	12
Ogon	11
Koboku	11
Tanjin	17
Chimo	15
Choji	12
Kujin	11
Keihi	11
Biwayo	9
Hop	10
Yobaihi	13
Enmeiso	15
Gobaishi	17
Sanshu	11
Shakuyaku	10

Kinnoshi	9
Chiyu	11
Maou	11

## Example 1

## Basic feed

Pressed barley	22.2 parts by weight
Corn	6.3 parts by weight
Beer residue	25.3 parts by weight
Vitamin & mineral agent	0.3 parts by weight
Italian rye grass, silage	28.5 parts by weight
Italian rye grass, dried	17.4 parts by weight



To the aforementioned basic feed, 1% of oren power by weight was added and was employed as a trial feed. The trial feed was given to 50 cows soon after delivery, which were 36~48 months old, for free consumption for a period of 300 days. Cows in a comparison zone were fed with the aforementioned basic feed instead.

During this feeding period, the number of cows which suffered from the bovine mastitis by staphylococcus aureus through outdoor infection was observed. The results show that the number of cows infected with bovine mastitis was 0 out of 50 in the trial zone whereas the number was 12 out of 50 in the comparison zone.

#### Examples 2-19

Test were conducted in the same manner as in Example 1 except that galenicals shown in Table 2 below were employed instead of oren.

The results are shown in the table below.

<u>Table 2</u>			
<u>Cows infected with bovine mastitis</u>			
<u>Examples</u>	<u>Name of galenicals</u>	<u>Trial zone</u>	<u>Comparison zone</u>
2	Kanzo	1/50	12/50
3	Ogon	1/50	12/50
4	Koboku	1/50	12/50
5	Tanjin	0/50	12/50
6	Chimo	0/50	12/50
7	Choji	1/50	12/50
8	Kujin	1/50	12/50
9	Keihi	1/50	12/50
10	Biwayo	1/50	12/50
11	Hop	1/50	12/50

12	Yobaihi	0/50	12/50
13	Enmeiso	0/50	12/50
14	Gobaishi	0/50	12/50
15	Sanshu	1/50	12/50
16	Shakuyaku	1/50	12/50
17	Kinnoshi	1/50	12/50
18	Chiyu	1/50	12/50
19	Maou	1/50	12/50

#### Example 20

1 kg Each of kanzo, oren, ogon, koboku, tanjin, chimo, choji, kujin, keihi, biwayo, hop, yobaihi, enmeiso, gobaishi, sanshu, shakuyaku, kinnoshi, chiyu and maou was placed in 10 liters of water respectively and extracted for 1 hour at the temperature of 100 centigrade. Each aqueous extract was diluted 1000 times to give an extract solution.

The aforementioned extract solution was administered instead of water to 50 heads of cows soon after delivery, which were 38 months to 48 months old. The same basic feed as in Example 1 was used.

Cows in a comparison zone were given water instead of the aforementioned extract solution.

As described above, the cows were fed for 10 months during which time, the number of cows which contracted bovine mastitis by staphylococcus aureus through outdoor infection was observed.

The results show that the number of cows infected with bovine mastitis in the trial zone was less than that in the comparison zone.

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